1002

Steroidal Sapogenins. XXXII.[1] Attempted Microbiological Oxidation

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Introduction

The successful microbiological hydroxylation of progesterone, Reichstein's substance S and similar steroids in position 11 (1–4) aroused our interest in the possibilities of similarly hydroxylating steroidal sapogenins. The hydroxylated derivatives, if obtained, could then be converted to Δ^{16}-20-ketopregnene derivatives (5) and thence elaborated to cortisone by well-known methods (6). As starting materials we selected the commonly occurring sapogenins, sarsasapogenin and diosgenin, and Δ^{4}-tigogenone prepared from the latter by Oppenauer oxidation.

Experimental Methods

Fermentation Procedure

The microorganisms used in the survey were obtained from the Culture Collection Unit of the Northern Utilization Research Branch.[4] The cultures were taken from lyophil tubes and grown on hay-infusion agar slants, usually for a period of 7 days. A loopful of cells or spores served as inoculum for 100 ml. of the fermentation medium contained in a 500-ml. Erlenmeyer flask. The medium em-

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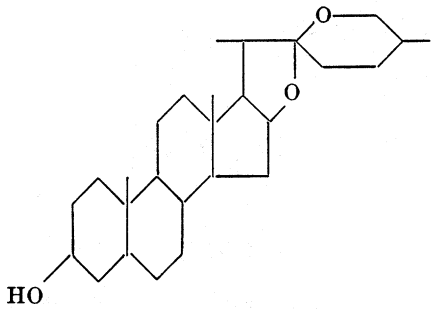
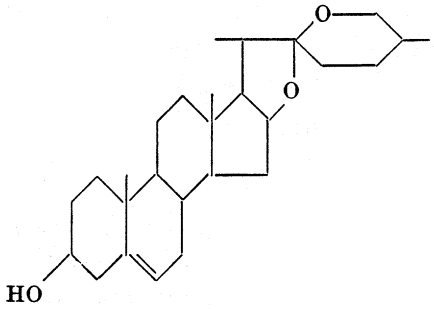
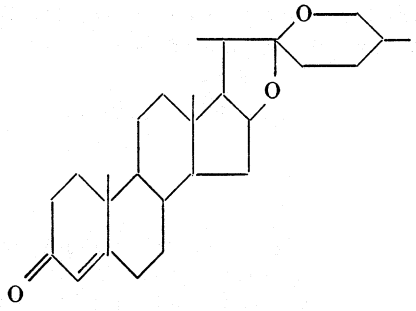
[4] We are indebted to Dr. C. W. Hesseltine for selecting and preparing the cultures.



sarsasapogenin



diosgenin



Δ^4 tigogenone

ployed in the survey was a modified Peterson and Murray medium (7, 8) and contained, per liter: technical glucose, 10 g.; lactalbumin,[5, 6] 5g., and corn steep liquor, 5 ml. The pH of the solution was adjusted to 5.2 with concentrated hydrochloric acid before sterilization. After sterilization at 121°C. for 30 min., the pH

[5] Edamin, an enzymatic digest of lactalbumin obtained from Sheffield Farms, New York, N. Y.

[6] The use of names of commercial products in this paper is solely for product identification.

was 4.5–4.8. The flasks were inoculated, incubated at 28°C., and agitated on a reciprocating shaker at the rate of eighty-eight 3-in. strokes/min. for 36 hr. After the initial growth phase, the sapogenin substrates were added to the flasks. The substrates were prepared by adding 25 mg. of the sapogenin to 2 ml. ethanol containing approximately 25 mg. polyoxyethylene sorbitan monooleate (Tween 80) and mildly heating on a steam bath to complete solution. The cultures were then incubated with agitation for an additional 36–48 hr. A few of the cultures which showed evidence of producing an oxygenated compound in preliminary tests were allowed to incubate as long as 20 days. After fermentation, the beers were sterilized at 121°C. for 30 min., transferred to sterilized bottles, and shipped to the Eastern Regional Research Laboratory for analysis.

Micro Qualitative Procedure for Sapogenins

The individual fermented samples containing 25 mg. sapogenin or fermentative product and mold mycelium in 100 ml. of medium were thoroughly mixed. A 12.5-ml. aliquot of the suspension was evaporated to dryness on the steam bath. The dried residue was broken up and extracted three times with hot benzene–methanol (1:1), using 20 ml. for each extraction. The combined extracts were filtered, and the filtrate was evaporated to dryness. The residue was dissolved in 5 ml. of chloroform. Aliquots equivalent to 300–600 μg. were spotted on Whatman No. 1 paper using a carbitol–methylcyclohexane system (8). After 18 hr. the sheets were dried and sprayed with phosphomolybdic acid reagent (9). Although we did not have a 3,11-dihydroxysapogenin available for reference purposes, it was noted that rockogenin, a 3,12- and chlorogenin, a 3,6-dihydroxysapogenin moved less than half the distance of monohydroxysapogenins. Consequently, all cases in which slower-moving spots were noted were tentatively considered as evidence that the sapogenins tested were being hydroxylated by the microorganism in question. Control experiments with media and various microorganisms in the absence of steroidal sapogenins always gave negative tests.

Macro Confirmatory Procedure for Sapogenins

Several techniques were used in larger scale runs designed to confirm definitely preliminary evidence of microbial hydroxylation of sapogenins. Thus in a typical experiment 2.0 g. of diosgenin in 7800 ml. was subjected to the action of *Agrobacterium radiobacter*. The aqueous portion was decanted from the bacterial cells and extracted with chloroform. No sapogenin was found. The cells were refluxed in acetone. The residue was oven dried, powdered, and extracted with chloroform in a Soxhlet extractor. The chloroform and acetone extracts were combined and evaporated to dryness, and the residue was saponified with methanolic potassium hydroxide. After dilution with water, the alkaline layer was extracted with ether–benzene (1:1). Following evaporation of solvent and crystallization from methanol, 1.6 g. of diosgenin, m.p. 205–207°, was obtained. The infrared spectrum was identical with that of an authentic specimen. No other steroid was found.

Several runs with various molds and Δ^4-tigogenone gave evidence of hydroxylation. Ten samples, each containing originally 25 mg. of sapogenin, were pooled, and the total contents were evaporated to dryness on the steam bath. After three extractions with hot benzene–methanol (1:1), the extracts were filtered, and the

TABLE I

Microorganisms Used in Attempts to Oxygenate Sapogenins

Bacteria
- *Acetobacter aceti*
- *Acetobacter suboxydans*
- *Agrobacterium radiobacter*
- *Bacillus megatherium*
- *Bacillus subtilis*
- *Cellulomonas biazotea*
- *Flavobacterium aurantiacum*
- *Mycoplana dimorpha*
- *Protaminobacter alboflavum*
- *Pseudomonas fluorescens*
- *Pseudomonas graveolens*
- *Pseudomonas indoloxidans*
- *Pseudomonas ovalis*
- *Pseudomonas putida*
- *Sarcina lutea*
- *Streptomyces albus*
- *Streptomyces parvus*
- *Streptomyces rutgersensis*
- *Streptomyces viridochromogenes*
- Undetermined B-1094
- *Xanthomonas campestris*

Yeasts
- *Candida guilliermondii*
- *Candida krusei*
- *Candida utilis*
- *Debaryomyces hansenii*
- *Hansenula anomala*
- *Kloeckera apiculata*
- *Nadsonia fulvescens*
- *Pichia membranaefaciens*
- *Rhodotorula glutinis*
- *Saccharomyces cerevisiae*
- *Saccharomyces fragilis*
- *Saccharomyces lactis*
- *Saccharomycodes ludwigii*
- *Schizosaccharomyces octosporus*
- *Torulopsis candida*

Molds
- *Absidia ramosa*
- *Actinomucor repens*
- *Alternaria* sp.
- *Aspergillus flavus*
- *Aspergillus fumigatus*
- *Aspergillus nidulans*
- *Aspergillus terreus*
- *Aspergillus unguis*
- *Aspergillus wentii*
- *Botrytis spectabilis*
- *Circinella umbellata*
- *Cladosporium herbarum*
- *Conidiobolus* sp.
- *Cunninghamella echinulata*
- *Dematium pullulans*
- *Eremascus albus*
- *Fusarium lycopersici*
- *Gliocladium catenulatum*
- *Mortierella isabellina*
- *Mortierella oligospora*
- *Mucor plumbeus*
- *Penicillium adametzi*
- *Penicillium expansum*
- *Penicillium funiculosum*
- *Penicillium javanicum*
- *Penicillium lanoso-coeruleum*
- *Penicillium lavendulum*
- *Penicillium lilacinum*
- *Penicillium roqueforti*
- *Penicillium vermiculatum*
- *Phycomyces blakesleeanus*
- *Pilaira anomala*
- *Rhizopus arrhizus*
- *Rhizopus nigricans*
- *Scopulariopsis brevicaulis*
- *Spicaria violacea*
- *Trichothecium roseum*
- *Ustilago zeae*

filtrate was concentrated under nitrogen. The residue was saponified by refluxing with methanol saturated with potassium hydroxide. After addition of water and ether–benzene extraction of the sapogenin, the solution was dried with anhydrous sodium sulfate and concentrated to dryness. The residue, 319 mg., was dissolved

in benzene–petroleum ether (1:1) and chromatographed on Florisil, 3.0 g. Elution with benzene–petroleum ether (1:1) yielded 124 mg. of material which gave several spots on a paper chromatogram. The infrared spectrum of this material indicated that no sapogenin or any other steroid was present. Further elution with benzene and benzene–chloroform (1:1) yielded 58 mg. of a fraction which contained Δ^4-tigogenone. No hydroxylation had taken place according to the infrared spectrum. Elution with chloroform and finally benzene–ethanol (10:1) yielded 76 mg. of a product which on paper chromatography showed the presence of a spot moving more slowly than Δ^4-tigogenone. The infrared spectrum showed absence of sapogenin, and steroids.

Results and Summary

A wide variety of microorganisms and culture conditions were employed in an attempt to hydroxylate sarsasapogenin, diosgenin, and Δ^4-tigogenone, three readily available sapogenins. Table I lists the bacteria, yeasts (10), and molds tested. Hydroxylation conditions similar to those used with progesterone were used, varying the culture conditions, such as aeration, agitation, inoculum, medium, pH of medium, and fermentation time. Although the sapogenins are insoluble in the medium, the suspension procedure described appeared to produce microscopic particles which did not settle out readily and showed no deleterious effects on the microorganisms.

Paper chromatography of solvent extracts in about 10% of the cases gave indications of substances moving more slowly than the original sapogenin. However, when these fermentations were repeated on the same or a larger scale, they yielded only original sapogenin or unidentified non-steroid products. Although one of the sapogenins tested had the Δ^4-3-keto moiety of progesterone, none of the organisms on any of the substrates produced a modified sapogenin. The conclusion must be made that steroidal sapogenins are not readily hydroxylated by microorganisms.

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